

Expression and epitope identification of myosin light chain isoform 1, an allergen in *Procambarus clarkii*

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ABSTRACT

Myosin light chain isoform 1 (MLC1) is reported to be a novel allergen in crayfish (*Procambarus clarkii*). However, little information is available about its allergic epitopes. In this study, recombinant crayfish MLC1 (rMLC1) was expressed and confirmed by mass spectrometry. Circular dichroic analysis and serological test were performed for the measuring of structural and immunological properties of rMLC1. Specific-protein-A-enriched IgG raised in rabbits against purified rMLC1 was used to screen a phage display random peptide library. Nine MLC1 mimotope clones were identified among 16 random clones after biopanning. Five conformational epitopes were identified with the program LocaPep, and mapped into 3 epitope regions at the antibody-binding interface of MLC1. MLC1 of crayfish showed high primary and secondary structure identity to MLC of other allergenic species, its epitopes were located in the structure conserved regions, and its cross-reactivity among related species was indicated by immunological assays.

1. Introduction

It is well known that shellfish plays an important role in human nutrition, the urge for a healthier diet has resulted in higher demands for shellfish (Venugopal & Gopakumar, 2017). Meanwhile, the increasing consumption of shellfish has been followed by increase in adverse reactions, and most of these are shellfish allergy mediated by immunoglobulin (Ig) E, which can result in diverse clinical symptoms (Faber et al., 2017). Unlike allergies caused by egg and milk, allergy to shellfish is typically a lifelong disorder that can significantly impair the quality of life (Jiménez-Saiz et al., 2017).

Allergenic shellfish comprise two invertebrate phyla of arthropods and mollusks, the phylum Arthropod contains the class Crustacea, which includes shrimp, prawn, crab, lobster, and crayfish (Wong, Tham, & Lee, 2019). The crayfish, short for red swamp crayfish (*Procambarus clarkii*) is a local favorite in China. For its appealing taste and good balance of nutrition, crayfish has become the most widely cultured freshwater shellfish species. The total production of crayfish in 2017 was about 1,129,708 tons, 36.59% higher than the 852,285 tons produced in 2016, and accounts for 35.05% of total freshwater shellfish production in China (Xu & Lv, 2018). The increased consumption of shellfish accompanied by higher frequency of food allergy, but over the

past decades, much of the attention was focused on the saltwater shellfish allergy, information on freshwater shellfish allergen was limited.

Hitherto, several allergens in crayfish muscle have been reported. The heat-stable myofibrillar protein tropomyosin is recognized as a major allergen of crayfish, and is considered as a cross-reactive allergen in shellfish (Faber et al., 2017). Another major allergen described in crayfish myosinogen is arginine kinase, which was reported to be involved in cross-reactivity among invertebrate (Chen et al., 2013; Yang, Hu et al., 2019; Yang, Liu et al., 2019). In recent years, other proteins including sarcoplasmic calcium-binding protein, triosephosphate isomerase, filamin C, and myosin light chain (MLC) have been identified as novel allergens in crayfish (Chen et al., 2013; Yang et al., 2017; Zhang et al., 2015).

In a previous study, Zhang et al. cloned two isoforms of MLC from crayfish muscle, which were designated as MLC isoform 1 (MLC1) and MLC isoform 2 (MLC2), respectively. The MLC2 is a homologue of the well documented MLC from white Pacific shrimp (*Litopenaeus vannamei*) and black tiger prawn (*Penaeus monodon*) (Zhang et al., 2015; Ayuso et al., 2008; Kamath et al., 2014). While the IgE-binding isoform detected in crayfish was the MLC1, which shows low protein sequence identity to MLC2. Therefore, the previously reported properties of L.

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vannamei MLC cannot provide sufficient information for crayfish MLC1 characterization. In spite of the physicochemical and digestion properties of crayfish MLC1 (Zhang et al., 2015), further investigation on its antigenicity is still insufficient, for its underrepresented percentage in crayfish muscle.

The antigenicity of an allergen depends on its physicochemical properties (e.g. thermal and pH stability) and epitopes. An epitope is the region on an allergen that is recognized and bound by specific antibodies (Liu & Sathe, 2018), consequently, the identification of epitopes in antibody-antigen interaction is a crucial step in the rational design of hypoallergenic food and immunotherapeutic strategies. Based on the location of amino acids and whether they are contiguous or not in a protein primary sequence, epitopes are typically categorized as either linear or conformational (Chen et al., 2016). Linear epitopes, which consist of continuous amino acids, can be identified by the fragmentation of the allergen, for example, the peptide microarrays can provide a tool for linear epitope mapping (Bernard et al., 2015). Conformational epitopes contain amino acids that are distributed discontinuously over the protein sequence, and come close to each other only when the protein formed a spatial structure (Chen et al., 2016). The most precise way to identify a conformational epitope is to determine the crystal structure of an antigen-antibody complex, which requires sophisticated techniques (Barba-Spaeth et al., 2016). An alternative approach to analyze the epitopes is to screen a phage display library with allergen specific antibodies to identify mimics of allergenic epitopes, called mimotopes. This latter method can overcome the high cost and time-consuming nature of peptide microarrays and crystal structure analysis, what's more, it can be applied in both linear and conformational epitope mapping (Chen & Dreskin, 2017).

From immunological studies, it is well known that shellfish are highly cross-reactive, homologous proteins such as tropomyosin and arginine kinase are responsible for the cross-reactivity (Broekman et al., 2017). Apart from major allergens, minor allergens such as sarcoplasmic calcium-binding protein and triosephosphate isomerase are recently reported to be cross-reactive (Nugraha et al., 2019; Xia et al., 2019). However, whether MLC1 is involved in cross-reactivity is not clear yet. Epitopes are known to be responsible for the immune reaction, thus the identification of epitopes is a necessary prerequisite for further understanding of the cross-reactivity of MLC1.

In spite of the increasing prevalence of crayfish allergy, few options are available for preventing allergic subjects from anaphylactic reaction, and avoidance of the offending food is the mainly treatment (Cabrera & Urra, 2015). Under these circumstances, there is an urgent need to create a more complete understanding of relevant allergens. In the present study, a recombinant MLC1 (rMLC1) was constructed to compensate for the difficulties in native protein purification and the low sensitivity of allergen in the crude extract. The epitopes of MLC1 were mapped with immunoscreening of a phage display random peptide library, and the cross-reactivity of MLC1 was evaluated according to the sequence homology, epitope distribution, and immunologic assays.

2. Materials and methods

2.1. Chemicals

The Eastep Super Total RNA Extraction Kit from Promega Biological (Shanghai, China) and the Gene Racer kit from Invitrogen (Karlsruhe, Germany) were used for cloning. Universal DNA Purification Kit and 3,3',5,5'-tetramethylbenzidine were from TIAN-GEN (Beijing, China). The DNA ligation kit for plasmid construction was from Takara (Kusatsu, Japan). The pEASY-T1 vector and DH5 α cells were purchased from TransGen (Beijing, China). The prokaryotic expression vector pET-28a(+) and *Escherichia coli* BL21 (DE3) competent cells were from Novagen (Madison, WI, USA). The Protein A Sepharose was from GE Healthcare (Waukesha, WI, USA). The Ph.D.-12 Phage Display Peptide

Library was purchased from New England BioLabs (Beverly, MA, USA). Goat anti-rabbit IgG-horseradish peroxidase (HRP) antibody and enhanced chemiluminescence (ECL) substrate were from Pierce (Rockford, IL, USA); goat anti-human IgE-HRP antibody and HRP-conjugated mouse anti-M13 phage antibody was from Abcam (Cambridge, UK). Freund's adjuvant used for allergen-specific IgG polyclonal antibody (pAb) preparation and tricaine methanesulfonate used for shellfish anesthesia were from Sigma Aldrich (Saint Louis, MO, USA). All the other reagents were of analytical grade.

2.2. Patient sera

Sera were obtained from 10 patients who had convincing histories of crayfish anaphylaxis with clear crayfish-exposure-related symptoms (No. Y64941, Y692603, Y694279, Y697811, Y718504, D843054, E481249, Y709270, D843749, and D839473) and 2 normal individuals (No. Q567125 and Q752168). All subjects voluntarily provided their sera with informed consent, and the sera were used with the permission of Xiamen Medical College Affiliated Second Hospital (human ethical approval number is XSH2012-EAN019, Xiamen, China). All sera were stored at -30°C until analysis.

2.3. Shellfish procurement and preparation

Live crayfish (*P. clarkii*), North Sea shrimp (*Crangon crangon*), white Pacific shrimp (*L. vannamei*), and black tiger prawn (*P. monodon*) were purchased from a local aquatic products market in Xiamen, China in June 2018. The obtained samples were handled with care by placing in polystyrene boxes, which were filled with water, twice that of the sample weight. The shellfish were immediately transported to Jimei University within approximately 30 min. On arrival to the laboratory, shellfish were subjected to washing using water ($\sim 20^{\circ}\text{C}$) to remove extraneous matter. Five random crayfish were anesthetized with tricaine methanesulfonate to collect muscle for total RNA extraction, and the samples used for myofibrillar protein preparation were kept in -80°C before experiments were conducted.

2.4. Preparation of the myofibrillar protein

Myofibrillar proteins from the shellfish muscle were prepared according to the method described by Zhang et al. (2015). Briefly, shellfish were thawed at 4°C , peeled, and beheaded, the muscle was then minced and homogenized with 20 mM phosphate buffer (pH 7.5) in a ratio of 1:10 (v/v), followed by centrifuged at $12,000 \times g$ for 10 min at 4°C . The precipitate was resuspended in 20 mM phosphate buffer (pH 7.5) and serially centrifuged 4 times. The last precipitation was the extracted myofibrillar protein, which was resuspended in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl.

2.5. Recombinant expression, purification, and identification of crayfish MLC1

Total RNA was isolated from *P. clarkii* using an Eastep Super Total RNA Extraction Kit. A rapid amplification of cDNA ends (5' RACE) was performed using the Gene Racer kit to obtain the 5' cDNA sequence. The above cDNA was treated as a template, and two pairs of primers designed from the already published MLC1 cDNA sequence (Acc. No. AFP95338.1) were used for a nested-PCR. The primer sequences are listed in Suppl. Table S1. The nested-PCR products were subsequently sub-cloned into a pEASY-T1 vector to construct a clone plasmid and expanded in DH5 α cells. All clones were sequenced by Bioray Corp. (Xiamen, Fujian, China).

The construction of MLC1 expression plasmid was followed the method of Hu et al. (2017). The primer pair MLC1-F and MLC1-R with digestion sites for the restriction enzymes *Nde* I and *Sal* I was used for PCR amplification (Suppl. Table S1). Restriction enzyme digested PCR

products were ligated into *Nde* I and *Sal* I restriction sites in pET-28a (+). The constructs (pET-28a-MLC1) were then transformed into *E. coli* BL21 (DE3) competent cells and cultured in Luria-Bertani medium, followed by induced with 1 mM isopropyl β -D-thiogalactoside (IPTG) (Hu et al., 2017). Cell pellets were harvested by centrifuged, re-suspended, and sonicated, rMLC1 was refolded by gradient dialysis from 4 M urea to 0 M urea (Mao et al., 2013). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the detection of the target protein. The purified rMLC1 band was excised from the stained SDS-PAGE gel and analyzed by Wininnovate Bio Corp. (Shenzhen, Guangdong, China) using a 5800 MALDI-TOF/TOF Analyzer from AB SCIEX (Boston, MA, USA).

2.6. Characterization of rMLC1

The structure examination of rMLC1 and comparison with that of native purified MLC was performed by measuring the far-ultraviolet circular dichroism (CD) spectra. The CD spectra were recorded from 260 to 190 nm on a CD spectrophotometer (Applied Photo Physics Ltd., Surrey, UK) using a 1-mm path length quartz cuvette (Li et al., 2018). Protein samples were adjusted to the concentration of 0.15 mg/mL, CD data were collected every 1.0 nm using an integration time of 0.25 s per step. To determine the melting temperature, temperature-dependent CD analysis was carried out at 220 nm with a temperature range of 20–80 °C and a rate of 1 °C/min. The secondary structures of the proteins were analyzed by the CDNN program (version 2.0).

For the activity validation of the recombinant protein, IgE-immunoblot and inhibition western blot were performed. The IgE-immunoblot was carried out as described by Hu et al. (2017). The purified rMLC1 was directly blotted on nitrocellulose membrane and allowed to dry, followed by blocking with 5% non-fat milk in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 8.0). The nitrocellulose membrane was then incubated with sera from crayfish allergic individuals or normal individuals (diluted 1:4) at 37 °C for 3 h. Inhibition western blot was carried out as described by Mao et al. (2013), the myofibrillar protein of crayfish was electrophoretically transferred onto nitrocellulose membrane after separated in an SDA-PAGE, and blocked with 5% non-fat milk in TBST. Patient sera (No. D843054, E481249, Y709270, D843749, and D839473) were mixed in an equal volume (diluted 1:5 in 20 mM PBS, pH 7.5), and pre-incubated with or without 5 μ g purified rMLC1 at room temperature for 3 h. The pre-incubated sera were then incubated with the nitrocellulose membranes for 3 h at 37 °C. Goat anti-human IgE-HRP (diluted 1:20,000) was used as the secondary antibody in IgE-immunoblot and inhibition western blot, and the results were visualized by ECL using the ECL substrate.

2.7. Preparation, purification, and titer analysis of rabbit anti-MLC1 pAb

The preparation of polyclonal rabbit anti-MLC1 antiserum was described in our previous research (Chen et al., 2013). The MLC1 specific IgG pAb was purified from the antiserum by affinity chromatography on Protein A Sepharose, and ELISA was carried out to analyze the titer of purified rabbit anti-MLC1 pAb. The fractions containing the purified IgG were collected and stored in –20 °C until use. Detailed information on antibody preparation and purification can be found in the Supplemental Section S1.

2.8. Biopanning a phage display random peptide library

For selection of mimotopes from the phage display library, 3 rounds of biopanning for affinity enrichment were carried out using a modified previously protocol harnessed by Yang et al., using the Ph.D.TM-12 Phage Display Peptide Library Kit, which displayed 10^{12} individual peptides (Chen & Dreskin, 2017; Yang, Hu et al., 2019; Yang, Liu et al., 2019). Overnight coating of the wells of ELISA plate at 4 °C with 150 μ L of purified rabbit anti-MLC1 pAb solution (100 μ g/mL in 0.1 M NaHCO₃,

pH 8.6) was used to capture IgG binding peptides. The phage solution ($\sim 2 \times 10^{11}$ phages in TBS, 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5) was added after the blocking incubation, and eluted after the washing steps.

The eluted phages were immediately used to infect freshly grown *E. coli* ER2738 for amplification, which were then concentrated with PEG-8000/NaCl precipitation and tittered according to the manufacture's specification. Then phage capture ELISA was performed as described by Chen & Dreskin (2017). Positive reactions were detected using HRP-conjugated mouse anti-M13 phage antibody (diluted 1:20) as the secondary antibody.

Single-stranded DNA from the selected clones was prepared according to the manufacturer's specifications of Ph.D.TM-12 phage display peptide library. The primer –96gIII (5'-GCCCTCATAGTTAGCGTA ACG-3') was used for automatic sequencing. The nucleotide sequences were then translated into amino acid to obtain the mimotope sequences.

2.9. Structure modeling and epitope mapping of MLC1

Linear epitope mapping was carried out by sequence alignment of selected mimotopes with one another or with the amino acid sequence of MLC1 using the DNAMAN (Lynnon Biosoft, San Ramon, CA, USA). For conformational epitope mapping, the three-dimensional (3D) structure was modeled on the website (<http://swissmodel.expasy.org/>) by homology modeling. The mimotope sequences obtained were calculated, analyzed, and mapped onto the 3D structure of MLC1 using the LocaPep (<http://atenea.montes.upm.es>) (Pacios et al., 2011). The resulting epitopes were marked to the secondary structure of MLC1 predicted by the PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>), and assigned to the 3D structure and visualized using the pyMOL (DeLano Scientific, San Carlos, CA, USA).

2.10. Cross-reactivity analysis among MLC1 of different species

For the cross-reactivity evaluation, primary and secondary structure of crayfish MLC1 was aligned with MLC from various allergenic species. Specialized BLAST to align sequences, available at the web server of national center for biotechnology information (NCBI, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>), was used to align the homologous sequences, and the homologous sequences from cross-reaction related species included in world health organization/international union of immunological societies (WHO/IUIS) Allergen Nomenclature website (<http://www.allergen.org/>) were recruited. Amino acid sequence alignment was prepared using Clustal Omega (<http://www.clustal.org/omega/>) (Sievers & Higgins, 2018), and then the multiple alignment file generated by Clustal Omega was uploaded to the online server ESPript 3.0 (<http://esprict.ibcp.fr/ESPript/ESPript/index.php>) for structure-based sequence alignment of the MLC1 (Robert & Gouet, 2014).

For the immunologic cross-reactivity analysis, western blot and inhibition western blot were performed. Myofibrillar proteins of 4 shellfish including *P. clarkii*, *C. crangon*, *L. vannamei*, and *P. monodon* were prepared according to the protocol of myofibrillar protein preparation described above. All the samples were loaded in the SDS-PAGE in quantities of 10 μ g/lane. The SDS-PAGE separated proteins were electrophoretically transferred onto nitrocellulose membranes and blocked with 5% non-fat milk in TBST. In western blot, the IgG-binding activities of the myofibrillar proteins were analyzed by using the rabbit anti-MLC1 pAb (1:10⁵ dilution) as the primary antibody and goat anti-rabbit IgG-HRP antibody (diluted 1:20,000) as the secondary antibody. For the inhibition western blot, pooled patient sera (No. D843054, E481249, Y709270, D843749, D839473, diluted 1:5) pre-incubated with or without 10 μ g crayfish myofibrillar protein was used as the primary antibodies, and then detected by goat anti-human IgE-HRP antibody (diluted 1:20,000).

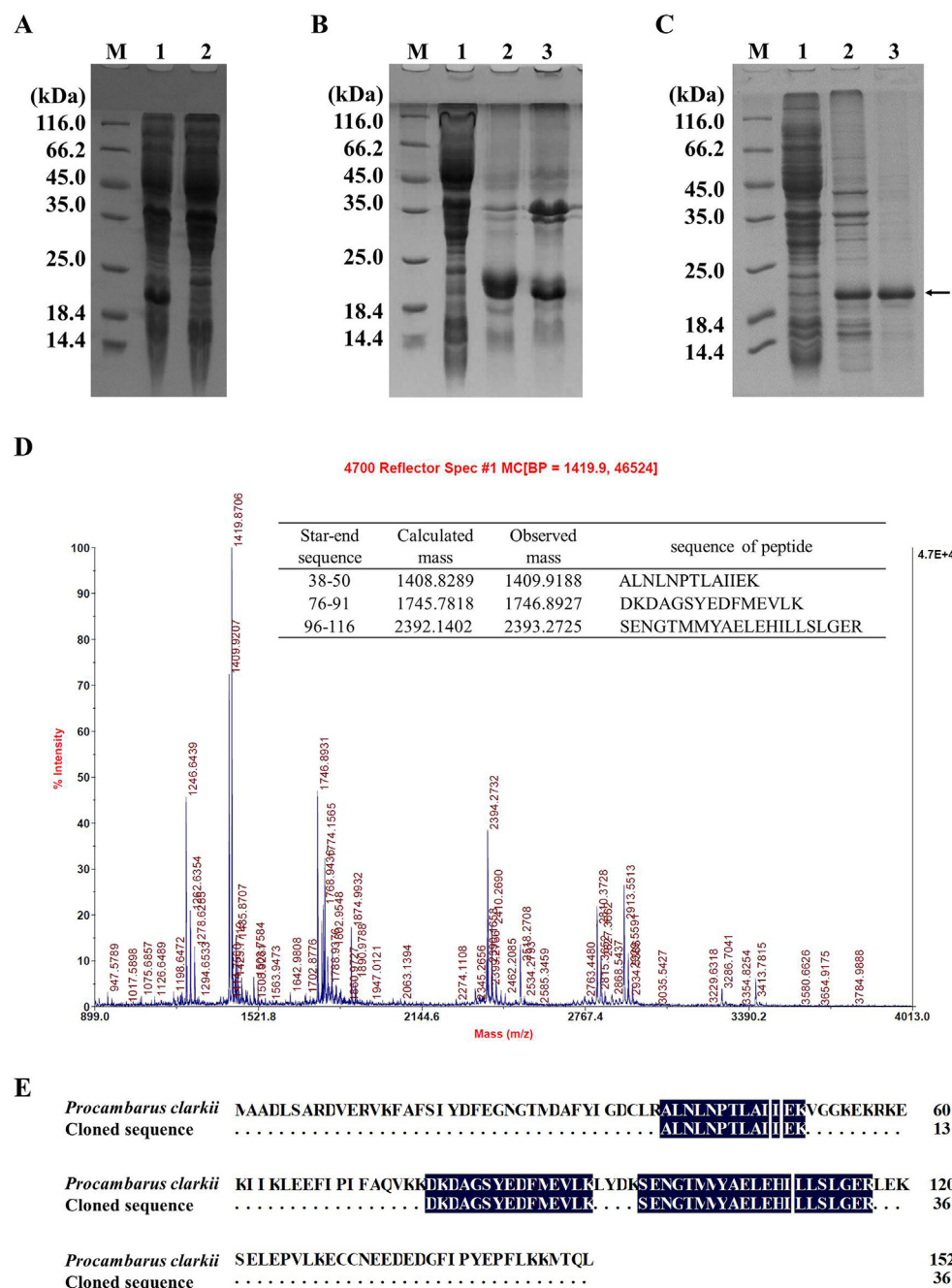


Fig. 1. Recombinant expression, purification, and mass spectrometry analysis of rMLC1 in *E. coli*. (A) SDS-PAGE analysis of expressed rMLC1 in *E. coli*. Lane M, protein marker; lane 1, the lysate from induced cells; lane 2, the empty vector of pET-28a. (B) SDS-PAGE analysis of the ultrasonicated fluid of the bacterial strain. Lane M, protein marker; lane 1, the ultrasonicated supernatants of the bacterial strain; lane 2, the supernatants of resuspended ultrasonicated sediment by 4 M urea; lane 3, the sediment of resuspended ultrasonicated sediment by 4 M urea. (C) SDS-PAGE analysis of the re-natured rMLC1. Lane M, protein marker; lane 1, the ultrasonicated supernatants of the bacterial strain; lane 2, the supernatants of refolded inclusion body by gradient dialysis from 4 M urea to 2 M urea; lane 3, the supernatants of refolded inclusion body by gradient dialysis from 2 M urea to 0 M urea. (D) Map of MALDI-TOF/TOF. (E) Protein sequence alignment between the recombinant protein and MLC1 from *P. clarkii*.

2.11. Statistical analysis

Data from the circular dichroism studies were presented as the mean $\pm$ SD. Data were analyzed by the General Linear Model and ANOVA of Duncan's test, differences between groups were considered significant when p values were < 0.05 . Each experiment was repeated at least three times.

3. Results

3.1. Expression, purification, and mass spectrometry analysis of the recombinant protein

The recombinant expression of plasmid pET-28a-MLC1 was sequenced, and the nucleotide sequence was proved to be totally matched with the documented sequence (Acc. No. AFP95338). The recombinant

strain was then expressed by inducing with IPTG, the molecular weight of rMLC1 was higher than the native *P. clarkii* MLC1 (Zhang et al., 2015), presumably because the $6 \times$ His-tag and 4 restriction enzyme cut sites (4.5 kDa) are carried (Fig. 1A). The recombinant protein was expressed as inclusion bodies that dissolved in solution buffer (20 mM Tris-HCl, 500 mM NaCl, 4 M urea, pH 8.0) (Fig. 1B), while it was easily refolded by gradient dialysis from 4 M urea to 0 M urea, and the target protein was purified during refolding (Fig. 1C).

To further confirm the amino acid sequence of the recombinant protein, the protein band was excised digested in-gel with trypsin, resulting peptides were analyzed by MALDI-TOF/TOF. The peptide mass fingerprinting of the protein (Fig. 1D) showed multiple peaks ranging from 900 to 4,000 Da, and peaks having signal-to-noise ratios of > 50 were analyzed by mass spectrometry/mass spectrometry. The effective peaks were compared with data in the NCBI database using the Mascot search tool, and the Mowse value was observed. Finally, three

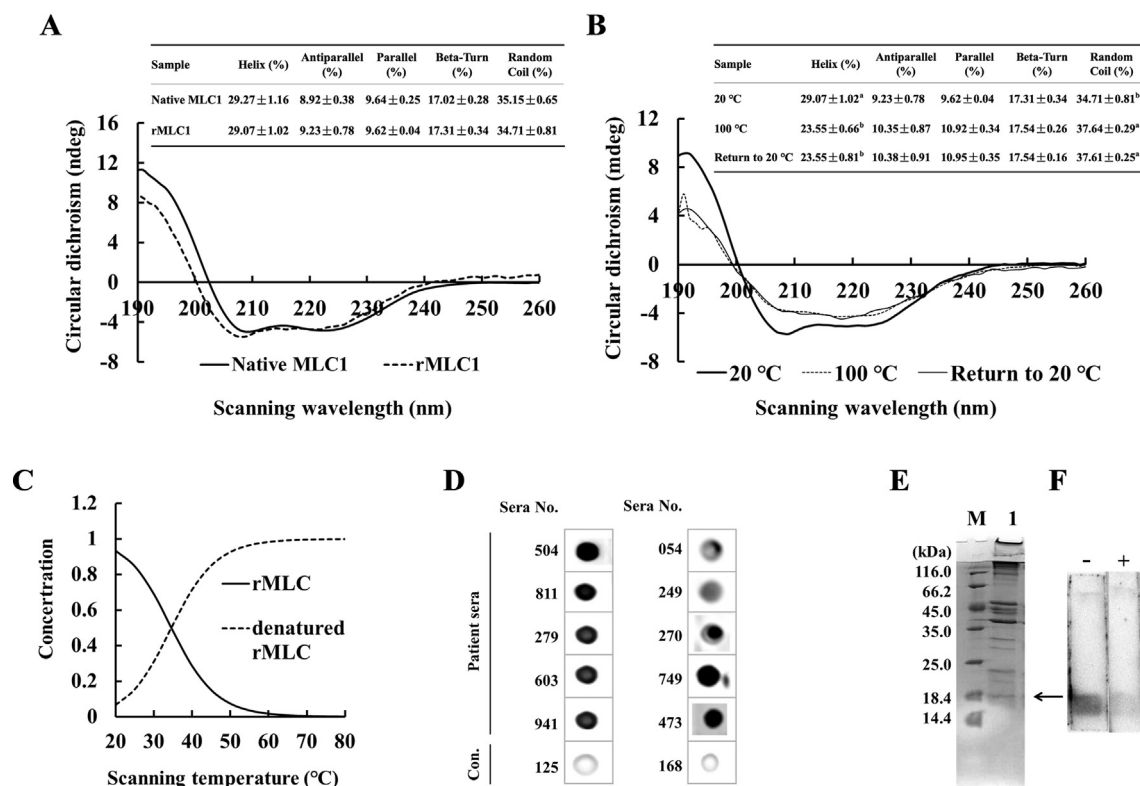


Fig. 2. Circular dichroism spectroscopy and immunoreactivity analysis of rMLC1. (A) Secondary structure analysis of native purified MLC1 and rMLC1 by CD spectroscopy. (B) CD spectral analysis of MLC1 subjected to different temperatures. Different superscript lowercase letters in the same row mean statistical differences ($p < 0.05$) by ANOVA of Duncan's test. No superscript lowercase letter means no statistical difference. (C) Determination of the thermal denaturation temperature value of rMLC1. (D) Immunoblot analysis of purified rMLC1 using sera from 10 crayfish allergic individuals and 2 normal individuals. (E) SDS-PAGE analysis of crayfish myofibrillar protein. Lane M, protein marker; lane 1, crayfish myofibrillar protein. (F) Inhibition western blot analysis of rMLC1. Nitrocellulose-blotted crayfish myofibrillar protein incubated with the pooled sera of crayfish allergic patients which were pre-incubated with (+) or without (-) purified rMLC1.

peptide that were 100% identical to the sequence of MLC1 from *P. clarkii* were obtained (Fig. 1E), indicating that the purified recombinant protein was MLC1 from *P. clarkii*.

3.2. Circular dichroism and immunoreactivity analysis of rMLC1

Far-UV CD spectra were examined to confirm the structure of purified rMLC1 and to compare the secondary structure of rMLC1 with native MLC1. The results of CD spectroscopic analysis showed that native MLC1 displayed two negative peaks around 208 and 222 nm, along with a positive peak around 190 nm (Fig. 2A), which is consistent with the secondary structure reported previously by Zhang et al. (2015). The spectrum of rMLC1 was similar to that of native MLC1, with slight difference (Fig. 2A), indicating a correct folding of the purified refolded rMLC1. The secondary structure of rMLC1 was 29.07% $\pm$ 1.02% α -helices, 18.85% $\pm$ 0.82% extended strands, 17.31% $\pm$ 0.34% β -turns, and 34.71% $\pm$ 0.81% random coils at 20 °C. However, high-temperature treatments had a noticeable effect on the secondary structure, significantly reduced the percentage of α -helix to 23.55% $\pm$ 0.66%, significantly increased the percentage of the random coils to 37.64% $\pm$ 0.19%, and partially increasing the extended strands and β -turns. In addition, the structure change is irreversible while the temperature returned to 20 °C (Fig. 2B). The denaturation temperature of rMLC1 is 34.6 $\pm$ 0.7 °C (Fig. 2C).

To investigate the IgE-binding activity of purified rMLC1, IgE-immunoblot using patients' sera were performed. Result of immunoblot showed that rMLC1 have strong IgE-binding activity with sera sample of crayfish allergic patients (Fig. 2D). Besides, the pooled sera from crayfish allergic patients were used to perform the inhibition western blot. As shown in Fig. 2E, the 18 kDa protein in crayfish myofibrillar

protein had a strong IgE-binding activity to patients' sera, while the binding activity was blocked after pre-incubation of the pooled patient sera with the purified rMLC1. These results revealed that the IgE-binding activity of rMLC1 was comparable to that of native MLC1. Thus, the rMLC1 obtained is consistent with native MLC1 in molecular weight, structure, and immunoreactivity.

3.3. Preparation and titer analysis of rabbit anti-MLC1 pAb

The purified rMLC1, with similar immunoreactivity and structural properties to the native MLC1, was then subcutaneously injected to the New Zealand rabbit, and antiserum with high specificity to rMLC1 was collected. The rabbit anti-MLC1 pAb was highly purified from the antiserum by column chromatography on Protein A Sepharose (Suppl. Fig. S1A). The bound protein eluted with 0.1 M glycine buffer (pH 3.0) was collected and analyzed by SDS-PAGE. Two bands with molecular mass of approximately 55 kDa and 25 kDa, which are consistent with the molecular weight of heavy chain and light chain of IgG, respectively, were isolated on SDS-PAGE (Suppl. Fig. S1B). Additionally, the result of ELISA proved that rabbit anti-MLC1 pAb with a titer of 1:10⁵ was purified (Suppl. Fig. S1C).

3.4. Phages isolated by biopanning with rabbit anti-MLC1 pAb

The purified anti-MLC1 pAb was then used as target protein for biopanning of the phage display random peptide library. Phages with binding activity to rabbit anti-MLC1 pAb were isolated from the phage random library after 3 rounds of biopanning, a total of 16 random clones were identified and sequenced (Table 1). Of the 16 clones, four clones displayed the same amino acid sequence of SNQHMNSTRPVA,

Table 1

DNA sequences and deduced mimotope sequences of positive clones derived from biopanning against rabbit anti-MLC1 pAb.

No.	Original nucleotide sequence (5'-3') ^a	Deduced mimotope sequence	Number of clone
1	AGTAATCAGCATATGAATAGTACTAGGCCGGTTGCT	SNQHMNSTRPVA	4
2	ATGGATCCGATGTATAAAGTCTGTCTTTATG	MDPMYNKSVPFM	4
3	CATTTGACGGCTACGGAGCTTGCGAATCTTATCAT	HLTATELANSYH	2
4	TGTCAGCAGCAGTATTATGCGGTTGGCATAATCCG	CQQQYYAVWHNP	1
5	AGGGATCCGATGTATAAAGTCTGTCTCTAATACG	RDPMYNKSVPYH	1
6	GAGGCTCCGCTGGAGTATTTCTTACTTCTATTTGG	EAPLEYFLTSIW	1
7	GAGCCGCTTCTTGGTCATGCGTTGGTCAGCTTATG	EPLLGHAFQQLM	1
8	TTTGTGAATAAGTCTCCGCTCTGAGACTTGGGCG	FVNKSPPPETWA	1
9	ACGGCTGTTTGGCTCCGACCGTGGCTTAATTG	TAVLAPQPWLNL	1

^a For deducing the amino acid sequences, the DNA sequences were read corresponds to the anticodon strand of the template.

four clones displayed the sequence of MDPMYNKSVPFM, and 2 displayed HLTATELANSYH, the rest 6 peptides were displayed by single clone. Thus, nine peptides were assigned as mimotopes of *P. clarkii* MLC1 in total.

3.5. Structural modeling and epitope determination of *P. clarkii* MLC1

The 3D structure of *P. clarkii* MLC1 was modeled based on its amino acid sequence by a homology model (PDB: 5W1A.1.B, 58.90% identity). The MLC1 structure shows a canonical fold with an N-terminal domain and a C-terminal domain. The N-terminal domain contains 4 α -helices, and the C-terminal domain contains 4 α -helices and 2 β -sheets (Fig. 3A). Overall, crayfish MLC1 is mainly composed of α -helices and random coils, this is corresponding with the results of the CD spectral analysis.

The conformational epitope of MLC1 was mapped using the LocaPep software, according to the theory proposed by Pacios et al., an epitope is assumed to form around a key residue, which is defined as a “seed” in the LocaPep software (Pacios et al., 2011). Thus, the surface of the MLC1 was scanned for key residues belonging to clusters, based on the mimotopes, five conformational epitopes was mapped around 4 key amino acids, T₄₅, Y₈₄, F₈₇, and N₉₉ (Table 2). The distribution of these epitopes on MLC1 were shown in Fig. 3B, the locations of these conformational epitopes could be mapped into 3 epitope regions at the antibody-binding interface: conformational epitope 1 (C-MLC1-1) formed region 1 around T₄₅, region 2 includes C-MLC1-2, C-MLC1-3, and C-MLC1-4 around Y₈₄ and F₈₇, and C-MLC1-5 formed region 3 around N₉₉ (Fig. 3C).

The linear epitope mapping by finding consensus sequences between the mimotopes and MLC1 molecule by DNAMAN was unsuccessful (data not shown), thus, no linear epitope was identified in the present study.

3.6. Cross-reactivity analysis of MLC1 among different species

To determine if MLC1 has the potential to induce cross-reaction among different species, the primary and secondary structure alignment of MLC1 among allergenic species including shellfish and insects was performed. To examine if the crayfish MLC1 shares the homology sequence with known MLC, the database of NCBI was searched for MLC from other species. Six other allergenic species included in the WHO/IUIS website listed under Animalia Arthropoda, including *Palaemon carinicauda*, *L. vannamei*, *C. crangon*, *Periplaneta americana*, *Coptotermes formosanus*, and *Bombyx mandarina*, were recruited (Suppl. Table S2).

Seven candidates including shrimp, termite, cockroach, and silk-worm pupa share a sequence identity of 72.25%, the most viable residues are mainly in the regions of amino acid (AA) 62–73, AA 120–129, and the C-terminal region (Fig. 4A). Secondary structure alignment of MLC1 also shows relatively high identity among species, the variable residue regions AA 62–73 and AA 120–129 are conserved in the secondary structure, with an identity of over 70% (Fig. 4A).

Experimental conformational epitopes of MLC1 identified in the present research were labeled in the aligned sequences using chromatic boxes, the majority of epitopes are distributed in the sequence and secondary structure conserved regions (Fig. 4A).

In order to confirm the cross-reactivity of MLC from different species, immunologic assays were performed using rabbit anti-MLC1 pAb and crayfish allergic patients' sera. The myofibrillar proteins of *P. clarkii*, *C. crangon*, *L. vannamei*, and *P. monodon* were subjected to SDS-PAGE, the MLC corresponding band around 18 kDa in the gel revealed its concentration in *L. vannamei* and *P. monodon* are lower than in *P. clarkii* and *C. crangon* (Fig. 4B). Despite of the concentration differences, the MLC corresponding band in all the tested myofibrillar proteins can be clearly detected by rabbit anti-MLC1 pAb (Fig. 4C). The 18 kDa protein in the myofibrillar proteins of these species also demonstrated IgE-binding activity to pooled sera from crayfish allergic patients (Fig. 4D). While pre-incubation of the pooled sera with crayfish myofibrillar protein resulted in complete inhibition of the IgE-binding activity to the 18 kDa protein in all the tested shellfish (Fig. 4D). In addition, a 38 kDa protein, which is corresponding to tropomyosin, was detected by the pooled sera (Fig. 4D).

4. Discussion

Shellfish is considered a common cause of IgE-mediated food allergy. Freshwater crayfish is the top-ranking aquaculture product in China, the increasing production and consumption have been accompanied by crayfish allergy, which has drawn public attention (Xu & Lv, 2018; Yang et al., 2017). In addition to the major allergens tropomyosin and arginine kinase, we have previously shown that MLC is a crayfish allergen for the specific recognition by IgE from shellfish allergic patients (Zhang et al., 2015). In the previously research, two isoforms of the MLC gene (MLC1 and MLC2) were cloned from crayfish, with a protein sequence homology of only 14% (Zhang et al., 2015). The identified allergenic isoform was MLC1, which share relatively high homology (83.66%) with *C. crangon* MLC (Cra c 5) (Kamath et al., 2014). Meanwhile, the amino acid sequence of crayfish MLC2 is 63% identical to the allergenic MLC from *L. vannamei* (Lit v 3) and *P. monodon* (Pen m 3) (Zhang et al., 2015). Apparently, more than one MLC molecules were involved in IgE-binding, allergenic MLC characterized in *L. vannamei* and *P. monodon* are the MLC2 isoform, while in *C. crangon* and crayfish, the reported MLC belong to the MLC1 isoform. Additionally, the overall frequencies of IgE antibody binding to MLC were different, the *L. vannamei* MLC was recognized by more than 50% of subjects with shrimp allergic subjects in the USA. However, the allergenic isoform recognized is MLC1 in Europe and China, and the IgE-binding rate is not as high as that of MLC2 in the USA (Zhang et al., 2015; Ayuso et al., 2008; Bauermeister et al., 2011). It seems that the designation as minor and major allergens are not necessarily absolute, it might be due to various influence factors, such as a different geographical background of the subjects, species difference, or other factors.

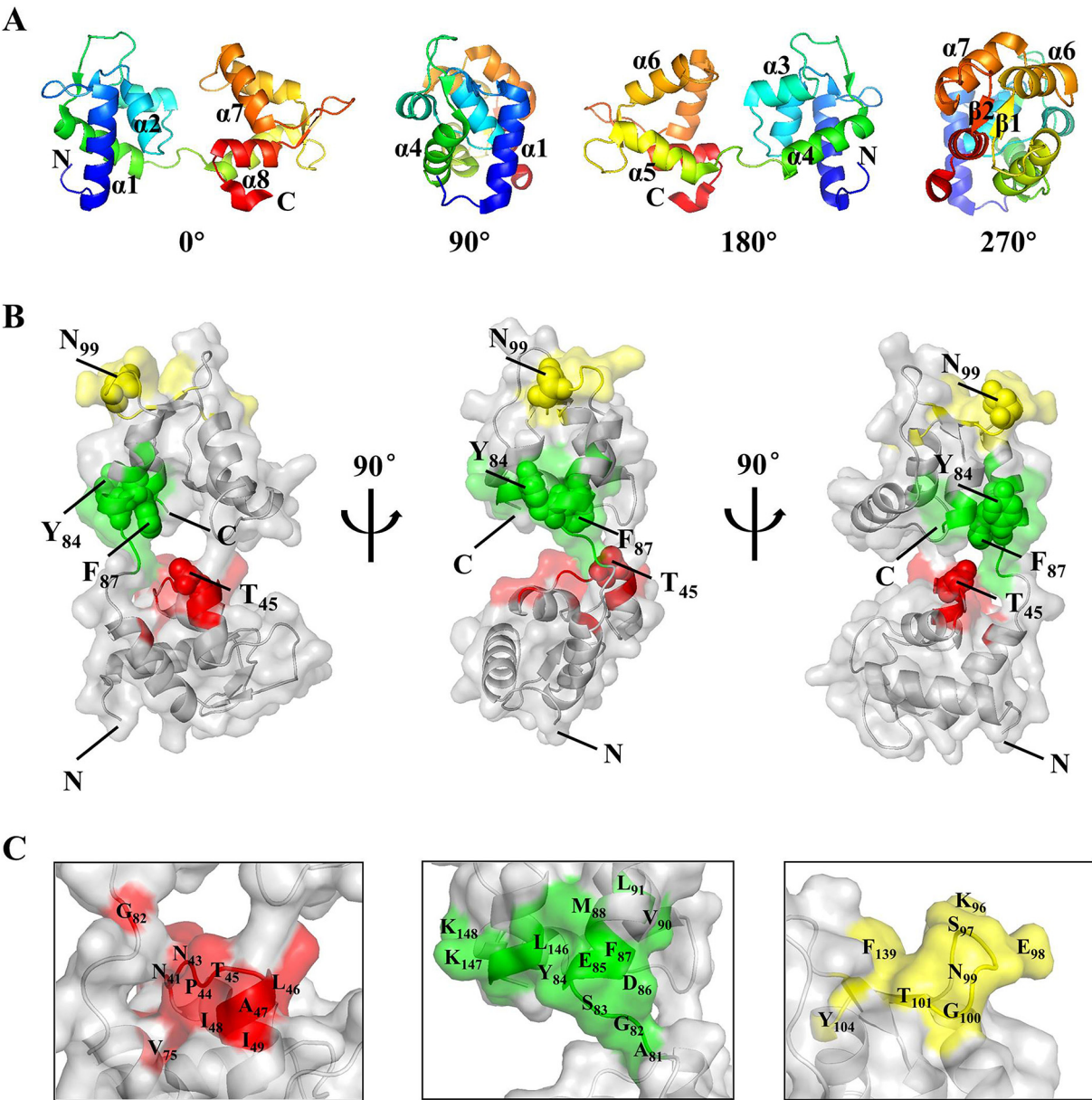


Fig. 3. Structure modeling and epitope mapping of *P. clarkii* MLC1. (A) Three-dimensional structure of MLC1. (B) Molecular graphics of the conformational epitopes computationally identified base on the mimotopes. The epitope regions 1–3 are highlighted in red, green, and yellow, respectively. The balls represent the key amino acid of each epitope. (C) Amino acids represent 3 epitope regions (1–3) of MLC1.

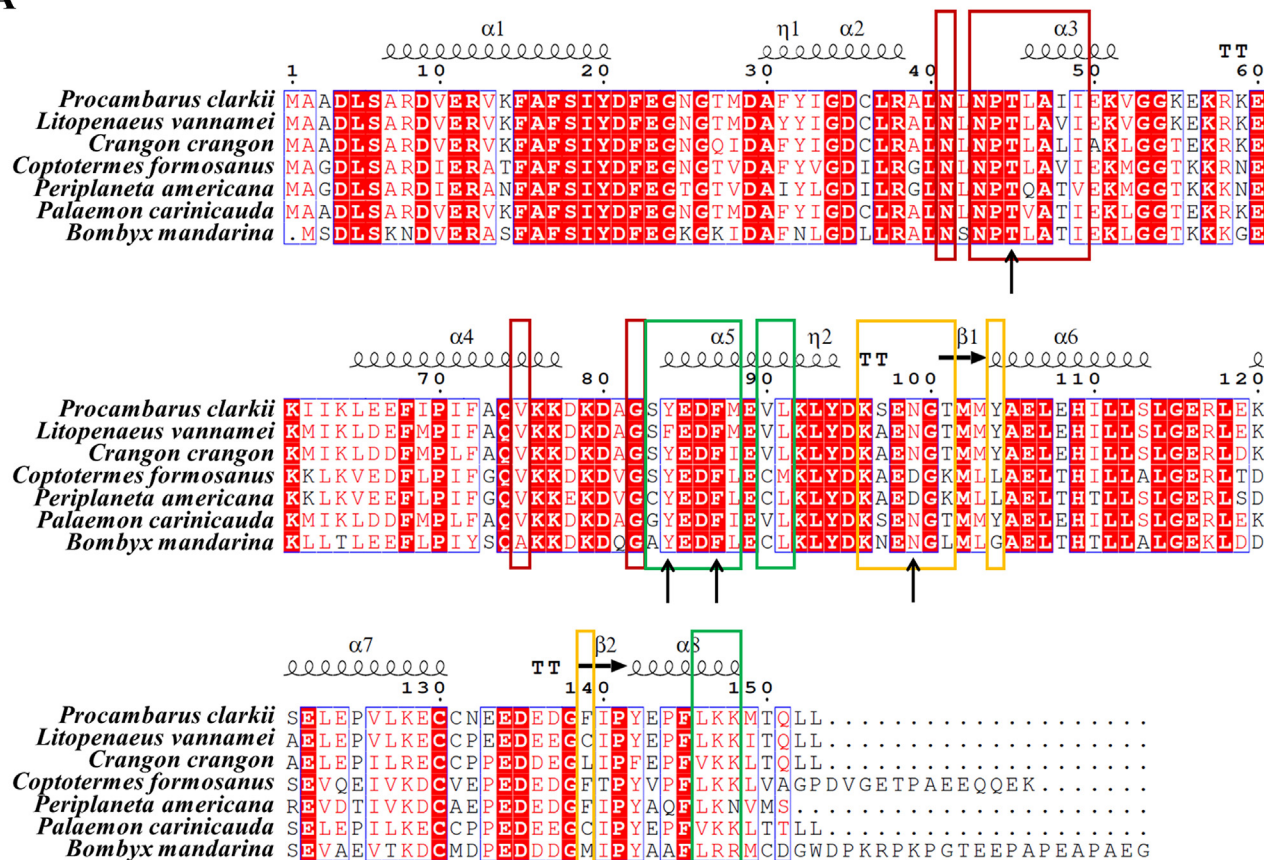
The previously identified MLC are mainly MLC2, IgE-binding epitopes of *L. vannamei* MLC have been well documented (Ayuso et al., 2008; Ayuso et al., 2010). While compared to MLC2, there is a limited knowledge on the epitopes of the MLC1. The native MLC1 purified from crayfish might be underrepresented in the investigation of antigenicity, due to low concentration in the source material or unfavorable conditions during extract preparation. In this study, the *E. coli* expression

vector pET-28a(+) was used for the MLC1 production. Although the recombinant protein was mainly expressed as inclusion bodies, it was easily renatured by gradient dialysis. Results of CD spectroscopic analysis confirmed that the rMLC1 is correctly folded. Besides, high-temperature treatment induced significant structure change of rMLC1, which is similar to the property of native MLC1 described previously (Zhang et al., 2015). Thereafter, the immunoreactivity of rMLC1 was

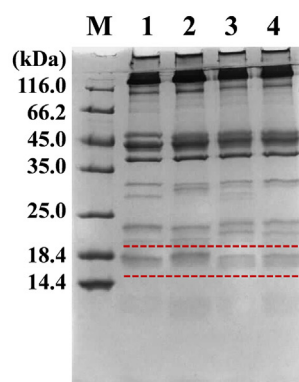
Table 2
Conformational epitopes of *P. Clarkii* MLC1 analyzed by LocaPep.

Name	Deduced mimotope sequence	Key amino acids	Amino acid residues and locations of epitope candidates	Epitope region
C-MLC1-1	TAVLAPQPWLNL	T ₄₅	N ₄₁ N ₄₃ P ₄₄ T ₄₅ L ₄₆ A ₄₇ I ₄₈ I ₄₉ V ₇₅ G ₈₂	1
C-MLC1-2	RDPMYNKSVPYH	Y ₈₄	S ₈₃ Y ₈₄ E ₈₅ F ₈₇ M ₈₈ L ₁₄₆ K ₁₄₇ K ₁₄₈	2
C-MLC1-3	EAPLEYFLTSLW	F ₈₇	G ₈₂ S ₈₃ Y ₈₄ E ₈₅ D ₈₆ F ₈₇ V ₉₀ L ₉₁ L ₁₄₆	2
C-MLC1-4	EPLLGHAFQQLM	F ₈₇	A ₈₁ G ₈₂ E ₈₅ F ₈₇ M ₈₈ V ₉₀ L ₉₁ L ₁₄₆	2
C-MLC1-5	FVNKSPPETWA	N ₉₉	K ₉₆ S ₉₇ E ₉₈ N ₉₉ G ₁₀₀ T ₁₀₁ Y ₁₀₄ F ₁₃₉	3

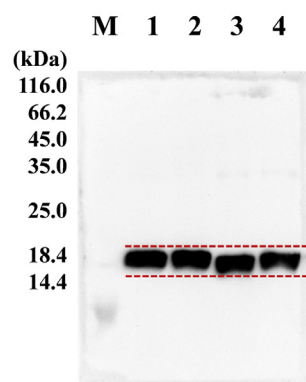
A



B



C



D

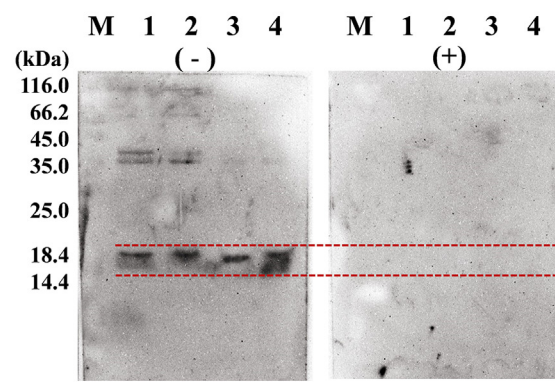


Fig. 4. Cross-reactivity analysis of MLC1 among allergenic species. (A) Sequence and secondary structure alignment of MLC from allergenic species. The secondary structure elements of MLC are shown on the top of the aligned sequences. Identical regions are colored red, and conserved regions (identity > 70%) are shown in boxes. Epitopes are designated by colored boxes on the MLC1 sequence. Regions of epitope regions 1–3 are marked by red, green, and yellow boxes, respectively. Key amino acids of epitopes are indicated with arrows. (B) SDS-PAGE analysis of myofibrillar proteins from different species. (C) Western blot analysis of MLC from different species using rabbit anti-MLC1 pAb. (D) Inhibition western blot analysis of MLC using pooled sera of crayfish allergic patients pre-incubated with (+) or without (–) crayfish myofibrillar protein. Lane M, protein marker; lane 1–4, myofibrillar proteins of *P. clarkii*, *C. crangon*, *L. vannamei*, and *P. monodon*, respectively.

demonstrated by the result of IgE-immunoblot. Besides, the result of inhibition western blot proved identical allergenic epitopes between native MLC1 and rMLC1. Similar immunological and structural properties suggesting the inclusion bodies were well renatured, which made it be able to prepare polyclonal antibody using rMLC1 as the antigen. Based on its specificity, the antibody prepared in the present work can be applied to screen the phage display random peptide library for the epitope mapping of crayfish MLC1.

Several methods have substantially improved their accuracy to

identify antigenic epitopes recently, such as the high-density peptide arrays for linear epitope screen, hydrogen/deuterium exchange mass spectrometry for conformational epitope mapping, and in silico epitope prediction for both linear and conformational epitopes (Ponce et al., 2019; Deng et al., 2017; Fu, Wang, Ni, Wang, & Wang, 2018). Epitope mimics using phage display technique allow determination of epitopes of individual patients, regardless of whether the epitopes are linear or conformational (Chen & Dreskin, 2017). In the present study, three conformational epitope regions of crayfish MLC1 were mapped by the

technique of phage display. The epitope region 3, formed by the C-MLC1-5, is partly overlapping with the predicted epitope region (AA 96–99) of crayfish MLC1 (Zhang et al., 2015). The region AA 76–83 was previously predicted as linear epitope region of MLC1 (Zhang et al., 2015), in the present case however, this region was included in the conformational epitope region 2. No linear epitope was determined in the present study, this is coinciding with the previously finding that conformational epitopes of MLC1 are predominantly recognized (Zhang et al., 2015).

In the establishing of processing methods for hypoallergenic products, the predominant epitope type of an allergen is an important factor that should be considered. For example, the predominant epitope type of *Scylla paramamosain* arginine kinase was conformational, and Maillard reaction can effectively reduce the sensitization of arginine kinase by glycosylated modifying the arginine and lysine in conformational epitopes (Hu et al., 2017; Han et al., 2018). In the identified epitopes of crayfish MLC1, three glycosylation sites were found, including the K₁₄₇ and K₁₄₈ distribute in epitope region 2, and the K₉₆ in epitope region 3. Besides, enzymatic cross-linking reaction is also an effective way to decrease the allergenicity of allergens. Tyrosinase and horseradish peroxidases are commonly used in shellfish allergen enzymatic cross-linking reaction. Tyrosinase catalyzes the oxidation of tyrosine, and results in oxidative cross-linking of tyrosine side chains, horseradish peroxidases act most on lysine, tyrosine, phenylalanine, or cysteine residues (Buchert et al., 2010). According to the amino acid component of epitopes of MLC1, lysine, tyrosine, and phenylalanine are contained in the epitope region 2 and 3. More importantly, the key amino acids of epitope region 2 are tyrosine and phenylalanine, indicating the potential of enzymatic cross-linking reaction in decreasing allergenicity of crayfish MLC1.

Generally speaking, biopanning for allergenic epitopes requires the corresponding IgE antibody from allergic suffers, for IgE antibody is a central player in food allergy (Chinthrajah, Hernandez, Boyd, Galli, & Nadeau, 2016). While recently, Groh et al. described the role of allergen-specific IgG antibody in blocking IgE-mediated hypersensitivity by competing with IgE for identical epitopes (Groh et al., 2017), which was in accordance with the conclusion that IgG has the ability to compete with IgE for allergen binding (Ponce et al., 2019; Orengo et al., 2018). Besides, our recently research proved that the IgE-epitopes of *S. paramamosain* arginine kinase are identical to its IgG-epitopes (Yang, Hu et al., 2019; Yang, Liu et al., 2019). These findings suggest that IgG could to some extent recognize the same epitope as IgE. Thus, rabbit anti-MLC1 IgG was used for the panning procedure to remedy the lack of specific IgE from shellfish allergic subjects. The IgG-epitopes determined in the present research might lay a foundation for the comprehensive analysis of the allergenic epitope of MLC1. However, in future studies, fundamental studies focus on IgE epitopes is essential as well.

There are some reports on cross-reactivity among shellfish and insects now, that is not surprising because they both belong to the Arthropoda (Wong et al., 2019; Broekman et al., 2017). Research on the cross-reactivity of tropomyosin and arginine kinase among shellfish and insects showed that high sequence homology and spatial structural similarity may lead to cross-reactivity among allergens (Yang, Hu et al., 2019; Yang, Liu et al., 2019; Gámez et al., 2014). To date, there is no published reports documenting cross-reactivity induced by MLC1, but due to the high sequence identity with *C. crangon* MLC, it seems that MLC1 has the potential to involve in cross-reactivity among species. The present research compared MLC from allergenic and cross-reactive related species, in addition to 4 shellfish and 2 asthma causing insects, an edible insect (*B. mandarina*) was recruited. Prevalence of insect food allergy in Europe is not known, while as a result of the cultural influences of traditional Chinese food and medicine, silkworm pupa is consumed in China, and anaphylaxis and cross-reactivity has been described upon ingestion (Broekman et al., 2017; Ji, Zhan, Chen, & Liu, 2008). MLC from allergenic species showed a high sequence

homologous to crayfish MLC1, and their secondary structures display relatively high identity, in parallel, the epitopes are distributed in the sequence and secondary structure conserved regions, revealing the foundation of the cross-reactivity of MLC1.

In this study MLC in different species were found to be reactive with IgG raised in rabbits against purified rMLC1, indicated the cross-reactivity of MLC among shellfish. It was also found that the crayfish myofibrillar protein was able to inhibit the IgE-binding activity of MLC from crayfish, North Sea shrimp, white Pacific shrimp, and black tiger prawn. These results provide additional proof of the cross-reactivity of crayfish MLC1. Additionally, the results of sequence and structure alignment verify the molecular foundation of cross-reactivity induced by MLC among shellfish and insect. To get a better understanding of the determinants relevant for MLC induced cross-reactivity, data on crystal structure and spatial structure superimposition among allergenic MLC from multiple species are required.

5. Conclusion

In the present study, a high yield recombinant crayfish MLC1 maintaining full IgE-binding activity and structural integrity was obtained, which might be used for further investigation in antigenicity or future diagnosis. Three conformational epitope regions were defined, and the structure conservation of epitope regions indicates the potential of MLC1 induced cross-reactivity, which was subsequently proved by the results of immunologic assays. As a consequence, comparing structural data from related species might allow for a more reliable basis for prediction of allergenic epitopes. These data lay the foundation for designing of mutated hypoallergenic form of MLC1 for developing hypoallergenic shellfish products or use as a non-allergenic vaccine.

CRediT authorship contribution statement

Yang Yang: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - original draft, Funding acquisition, Visualization. **Hui-Fang Yan:** Investigation, Data curation. **Yong-Xia Zhang:** Conceptualization, Methodology. **Heng-Li Chen:** Methodology. **Min-Jie Cao:** Supervision, Writing - review & editing. **Meng-Si Li:** Methodology. **Ming-Li Zhang:** Resources. **Xin-Rong He:** Investigation. **Guang-Ming Liu:** Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2020.126422>.

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Expression and Epitope Identification of Myosin Light Chain Isoform 1, an Allergen in *Procambarus clarkii*

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Table S1. Specific primer sequences used for the amplification of MLC gene from *P. clarkii* ^a.

Primer	Sequence (5'-3')
MLC-F1	CCGTTAACGTGGTGTGCTAGA
MLC-R1	CGGACACAAGACACATACGGA
MLC-F2	TTAACGTGGTGTGCTAGAGGC
MLC-R2	GTTTGGTCGAGCCCACAGA
MLC-F	<u>CATATG</u> ATGGCCGCGGATCTCAGTG (containing the <i>Nde</i> I site)
MLC-R	<u>GTCGAC</u> GAGTAGTTGAGTCATCTTCTTAAGG (containing the <i>Sal</i> I site)

^a The underlined nucleotides are restriction enzyme cutting sites.

Table S2. Comparison of MLC1 in allergenic species.

Species	MW (kDa)	Amino acid length	Genbank accession No.
Red swamp crayfish (<i>Procambarus clarkii</i>)	18	153	AFP95338
Ridgetail white prawn (<i>Palaemon carinicauda</i>)	18	153	AYC12379
White Pacific shrimp (<i>Litopenaeus vannamei</i>)	18	153	XP_027231019
North Sea shrimp (<i>Crangon crangon</i>)	18	153	ACR43477
American cockroach (<i>Periplaneta americana</i>)	18	151	AEV23877
Termite (<i>Coptotermes formosanus</i>)	19	166	AGM32403
Silkworm pupa (<i>Bombyx mandarina</i>)	18	150	XP_028042264

S1. Preparation, purification, and titer analysis of rabbit anti-MLC1 pAb

The purified rMLC1 was diluted to a concentration of 200 µg/mL and emulsified with complete Freund's adjuvant in a ratio of 1:1 (v/v). One milliliter of the mixture was then subcutaneously injected into the adult female New Zealand white rabbit. Further injections were carried out at 14, 28, and 35 days later, using the mixture of purified recombinant protein and incomplete Freund's adjuvant. The blood was collected from the retroorbital plexus of the rabbit 5 days after the last injection, and centrifugated to obtain the antiserum.

The MLC1 specific IgG pAb was purified from the antiserum by affinity chromatography on Protein A Sepharose. The antiserum was mixed with 0.1 M Tris-HCl buffer (pH 8.0) in a ratio of 1:1 (v/v), and subsequently applied to a Protein A Sepharose (ø 1 cm×5 cm) pre-equilibrated with 0.1 M Tris-HCl buffer (pH 8.0). Binding proteins were eluted using 0.1 M glycine buffer (pH 3.0) at a flow rate of 1 mL/min, and neutralization by 1 M Tris-HCl buffer (pH 8.0). Fractions containing the target protein were detected by SDS-PAGE.

ELISA was carried out to analyze the titer of purified rabbit anti-MLC1 pAb. briefly, ELISA plates (Nunc Maxisorb, Copenhagen, Denmark) were coated overnight with 200 ng/well of purified rMLC1 and blocked with 5% non-fat milk. The wells were then incubated with the serially diluted purified antibodies. To detect the antibodies, goat anti-rabbit IgG-HRP antibody (diluted 1:2,000), was used as the secondary antibody. The samples were then incubated with 3,3',5,5'-tetramethylbenzidine at 37 °C for 20 min and the reaction terminated with 2

M H₂SO₄. The absorbance at 450 nm was measured on an automated ELISA platereader (Bio-Rad, Philadelphia, PA, USA). All the incubation steps were performed at 37 °C for 2 h.

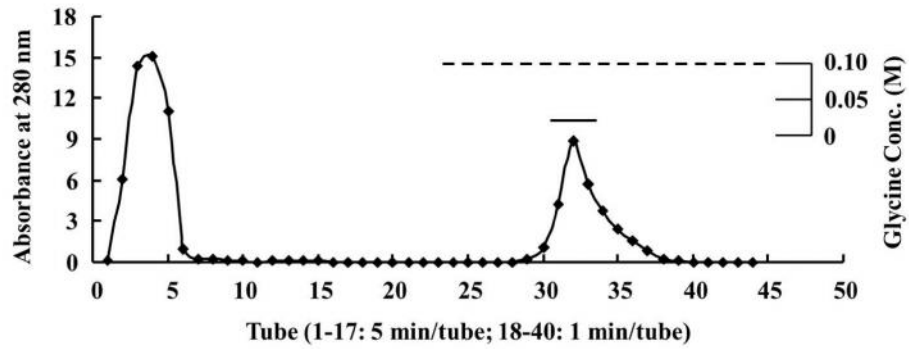
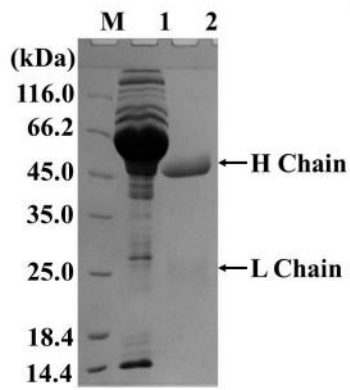
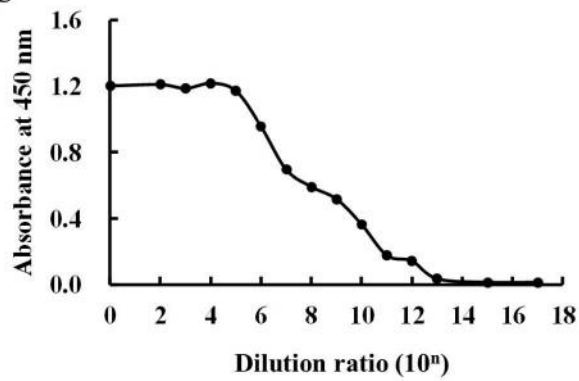
A**B****C**

Fig. S1. Purification and titer analysis of rabbit anti-MLC1 pAb.

(A) Protein A Sepharose chromatography profile. Target protein fractions under the bar were pooled (lane 2 in B). (B) SDS-PAGE of purified IgG pAb. Lane M, protein marker; lane 1, polyclonal rabbit anti-MLC1 antiserum; lane 2, purified IgG pAb from antiserum. (C) Titer analysis of purified IgG pAb by ELISA.